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MECHANISM OF ACTIVATION AND SPECTRAL SHIFT OF THE F-695 EMISSION BAND IN CHLOROPLASTS AS INDUCED BY 1,10-PHENANTHROLINE

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SUMMARY

1. Changes in the fluorescence emission spectrum of chloroplast, at 77 °K, induced by chaotropic reagents and 1,10-phenanthroline, were analyzed.

2. Fourth-derivative analysis of the emission spectra identified the exact location of a new band (referred to as "F-700") at 700 nm and showed that the conversion of F-695 into F-700 does not occur by a gradual red-shift of the F-695 band, but by the appearance of a new band at 700 nm at the expense of an intensity decrease in the F-695 emission.

3. F-700 shows two distinct fluorescence characteristics, namely the wavelength of its emission maximum and its intensity, but still retains the principal properties of F-695 such as steep temperature dependence at low temperatures, transient phenomena at 77 °K, and an excitation spectrum of the Photosystem II type. Thus F-700 is concluded to be a modified state of F-695.

4. In addition to the compounds of the urea–guanidine class, inorganic anions such as SCN^-, I^- and ClO_4^- were active in the transformation. The specificity and theorder of effectiveness of these reagents indicated that their action is that of chaotropic reagents. Transformation was inhibited by the presence of compounds such as sugars, salts, alcohols and dimethylsulfoxide which seem to affect the activity of water.

5. 5-Methyl-1,10-phenanthroline partly substituted for the action of 1,10-phenanthroline, while the other six different derivatives of 1,10-phenanthroline and a few other bifunctional ligands were inactive. The structure–activity relations and the effective concentrations in the transformation differed greatly from those of the inhibition of the electron transport chain, suggesting that the action of 1,10-phenanthroline in the transformation is a yet unrecognized action of this reagent on Photosystem II.

6. Transformation was generally observed in chloroplast preparations from 11 different higher plants and two species of algae tested. In *Lolium* sp. the transformation was partly attained by 1,10-phenanthroline alone.

7. From these results, the state of F-695 in chloroplast membranes and the mechanism of transformation into F-700 are discussed.

INTRODUCTION

In 1960, an emission band with a maximum at a wavelength near 695 nm, at 77 °K ,was first observed in bean leaves by Litvin et al. [1]. Many workers have reported a similar low-temperature emission band in intact cells or chloroplast preparations from various chlorophyll *a* containing organisms [2–9]. With respect to the relation of the emission component (F-695) to the photosynthetic meachnism, it has been proposed that F-695 is associated with the energy traps of Photosystem II [2, 3, 5, 10, 11]. Experimental support for the proposal has the following nature: (a) appearance of the F-695 band when photosynthesis is light saturated [12]; (b) photoinitiation of the band at 77 °K [2]; (c) preferential sensitization of the emission by pigments of Photosystem II [7, 13] and localization in subchloroplast Particle II [14–21]; (d) parallel disappearance and reappearance of the emission band with the activity of C-550 photooxidation in an extraction–reconstitution experiment [22]; and (e) sensitvity of the emission band to the functional environments [16–18, 23–25], especially to the redox state of a component associated with the oxidizing side of Photosystem II [23, 25]. This hypothesis, however, is not yet firmly established. The primary nature of the fluorescence-yield changes in the F-695 emission has been called into question [26, 27].

On the other hand, the F-695 emission has been shown to have some unique properties in chlorophyll fluorescence in vivo: (a) the emission band normally appears only at temperatures below 150 °K [13, 28–31]; and (b) the emission is especially sensitive to various additions such as dimethylsulfoxide [30], compounds of the urea–guanidine class [32], and a naturally occurring proteinaceous factor from *Ricinus* [33,34]. Recently, the author also reported a marked reversible activation and spectral shift of the F-695 emission with the addition of urea and 1,10-phenanthroline to spinach chloroplasts [32]. These unique properties of the F-695 emission band are as yet poorly understood, but appear to related to the specific molecular environments in chloroplast membranes and to the specific function of this pigment in the photosynthetic mechanism.

The present study was intended to analyze the mechanism of the specific action of urea and 1,10-phenanthroline on F-695 to elucidate the molecular environments and the function of the pigment in vivo.

MATERIALS AND METHODS

Preparation of chloroplasts fragments

Chloroplast fragments from spinach and other higher plants were prepared by the method described earlier [35].

Measurement of fluorescence emission spectra

Fluorescence emission spectra were measured by an instrument described in the previous report [32]. Fluorescence was excited at 431 nm (interference filter, half-band width of 15.5 nm). The fluorescence emitted was analyzed by a grating monochromator (band width of 2.5 nm, but 5.0 nm where indicated). The emission spectra were corrected, where indicated, to allow for the spectral sensitivity of the photomultiplier (EMI 9659QB, extended S-20) and the transmission efficiency of the

monochromator. A correction factor for changes in photomultiplier sensitivity and monochromator transmission with wavelength was obtained by checking the experimental setup with a calibrated standard lamp (EPIR-1093, Eppley Lab., Inc.). The fourth derivatives of the fluorescence emission spectra were calculated with a digital computer (NEAC 2200-500). The chloroplast concentrations of samples used for the measurements were 10 μg chlorophyll/ml.

Measurement of fluorescence excitation spectra

The light for fluorescence excitation was obtained from a tungsten–halogen lamp (45 W, Sylvania) provided with a combination of a Bausch and Lomb grating monochromator (band width, 5.0 nm) and filters; heat absorbing filters (HA-50, Hoya) and a ultraviolet absorbing filter (UV-39, Toshiba). The fluorescence was collected from the same surface which received the excitation light. Each fluorescence band was isolated through a combination of interference filters and a cut-off filter: (a) V-R67 (Toshiba), KL-68 (Toshiba), and 2145 (Nihon Shinku Kogaku; peak wavelength, 682 nm; half-band width, 7.5 nm) for F-685 band; (b) V-R69 (Toshiba) and 2146 (Nihon Shinku Kogaku; peak wavelength, 702 nm; half-band width, 9.0 nm) for F-695 or F-700 (see Results) band. An EMI photomultiplier (9659QB) was used as the light detector; the response signal was amplified and recorded on a strip chart servo recorder. Excitation spectra were corrected for variations in the incident light intensity as measured with a radiometer (MPM-1, Japan Spectroscopic Co. Ltd.).

Reagents

Reagents were purchased from the following pharmaceutical companies: guaranteed reagents of 2,9-dimethyl-1,10-phenanthroline, 1,10-phenanthroline, 4,7-diphenyl-1,10-phenanthroline, 5-nitro-1,10-phenanthroline, pyridine, 2,2′-dipyridine, 4,4′-dipyridine, 1,3-diazine, 1,4-diazine, 2,4,6-tris-(2-pyridyl)-*s*-triazine, 3,3′-diaminobenzidine tetrahydrochloride, *o*-phenylenediamine and the S.U. grade of guanidine hydrochloride from the Tokyo Chemical Industry Co., Ltd (Tokyo, Japan); guaranteed reagents of 4,7-dimethyl-1,10-phenanthroline, 5-methyl-1,10-phenanthroline, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline sulfonate (sodium salt) and 4,7-diphenyl-1,10-phenanthroline sulfonate (sodium salt) from the Dojindo Co., Ltd, Research Lab. (Kumamoto, Japan); guaranteed reagents of KSCN, KI, glucose, and sucrose from Wako Pure Chemical Industries, Ltd (Osaka, Japan); guaranteed reagents of urea and other reagents from Ishizu Pharmaceutical Co., Ltd (Osaka, Japan).

RESULTS

Spectral changes

As described previously [32], the low-temperature fluorescence emission spectrum of spinach chloroplasts, excited by light absorbed by photosynthetic pigments, changes markedly with the addition of guanidine hydrochloride and 1,10-phenanthroline. Fig. 1 shows the corrected (for the spectral sensitivity of the detector and the transmission efficiency of the monochromator) fluorescence emission spectra from 650–800 nm (plotted against wave number) of spinach chloroplasts in the presence or

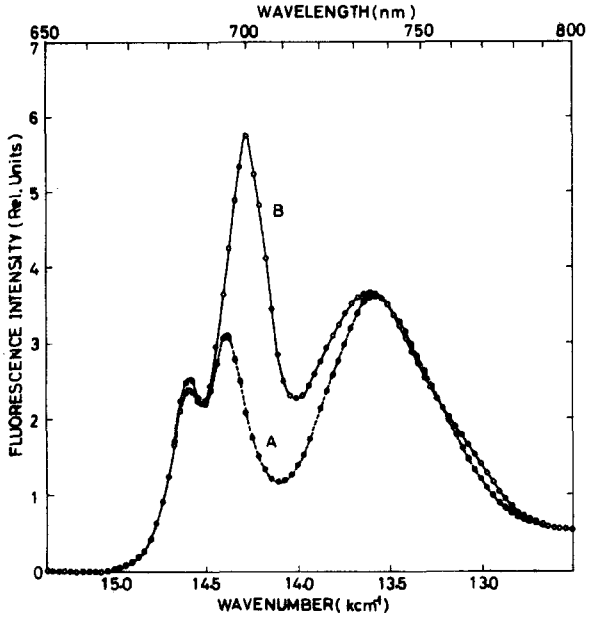

Fig. 1. Corrected fluorescence emission spectra of spinach chloroplasts, at 77 °K, in the absence (A) and in the presence (B) of 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. Chloroplast preparations were suspended in 50 mM Tris–HCl (pH 7.2) in water.

in the absence of 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. The emission spectrum in the presence of guanidine hydrochloride and 1,10-phenanthroline is quite different from that of the control sample in three respects. First, the location of the second emission maximum, i.e. the F-695 emission band, is shifted from a wavelength near 695 nm to about 700 nm. The second distinctive characteristic of the fluorescence emission spectrum is a marked activation (nearly doubled) of the new band near 700 nm, as compared with the F-695 band in the original sample. Thirdly, the intensity of the first emission maximum, i.e. the F-685 emission band, does not shift, but is partly reduced in accompaniment with the activation of the new band. In addition to the above-mentioned three features, some changes in the long wavelength emission band, i.e. the F-735 band, are evident, but are not analyzed further in this paper.

Higher derivatives of absorption spectra were used to determine the peak positions of the contributing absorption bands [36–38]. This principle was introduced to determine the exact position of the new emission band (referred to as "F-700"). Fluorescence intensities were digitized at every 25/3 cm^{-1}, fed into a digital computer and the fourth-derivative values from 650–720 nm were computed using 16 different digitized data points with derivative intervals of 3.2–2.0 nm. The fourth-derivative curves thus obtained, for the fluorescence emission spectra shown in Fig. 1, are pictured in Fig. 2. The principal emission bands in the control sample were F-685 (684.0–685.9 nm) and F-695 (694.0–694.7 nm). No emission band at a point near 700 nm is observed in the control sample (Fig. 2-A). On the other hand, a new emission maximum at a point near 700 nm (700.1–700.5 nm) appears along with the decre-

ase in the F-695 emission in the presence of guanidine hydrochloride and 1,10-phenanthroline (Fig. 2-B). Guanidine hydrochloride alone caused the reduction of the intensity of the F-695 emission to about 40 % of the control (estimated by the height of the fourth-derivative spectra), but did not cause any wavelength shift in the emission band (data not shown). On the further addition of 1,10-phenanthroline to the sample, the intensity of the F-695 emission decreased along with the appearance and increase of the F-700 emission band; F-700 is not produced by a gradual red-shift of F-695, but arises at the expense of the intensity decrease in the F-695 emission.

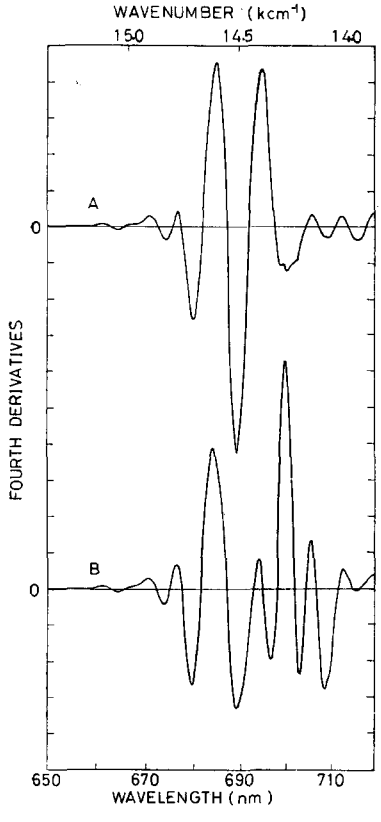

Fig. 2. Fourth-derivative curves of the corrected fluorescence emission spectra shown in Fig. 1.

If there is no change in the form of the distribution function or in the dispersion of the distribution of the fluorescence emission spectrum, the height of the fourth-derivative spectra should be proportional to the intensity of the original band. The intensity of the F-695 emission in the fourth-derivative spectrum is lowered about 15 % in Fig. 2 in the presence of guanidine hydrochloride and 1,10-phenanthroline (see also Fig. 1).

Effects of inorganic anions

In the previous paper, compounds of the urea–guanidine class such as guanidine hydrochloride, methylguanidine hydrochloride, urea, *N*-methylurea, and thio-

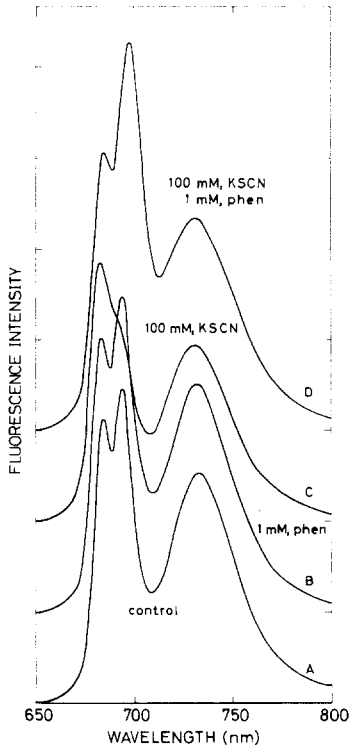

Fig. 3. Fluorescence emission spectra of spinach chloroplasts, at 77 °K, in the presence of 100 mM KSCN and 1 mM 1,10-phenanthroline: A, no additions; B, 1 mM 1,10-phenanthroline; C, 100 mM KSCN; D, 100 mM KSCN and 1 mM 1,10-phenanthroline. Chloroplast preparations were suspended in 50 mM Tris–HCl (pH 7.2) in water. For clarity, the base lines are successively displaced.

urea were shown to be capable of producing an activation and wavelength shift in the F-695 emission if they were added to chloroplasts along with 1,10-phenanthroline [32]. It becomes clear in this paper that some inorganic anions at relatively low concentrations are also effective in producing these changes. They include SCN^-, I^- and ClO_4^-. Fig. 3 shows the quenching and the activation effects of KSCN on the uncorrected fluorescence emission spectra of spinach chloroplasts at 77 °K. Potassium thiocyanate alone has a quenching effect as is seen also in the case of urea or guanidine hydrochloride, but the effect is somewhat more marked than that produced by the latter two. By the joint addition of KSCN and 1,10-phenanthroline, the F-695 emission is activated and red-shifted also as in the case of urea or guanidine hydrochloride. The effects of various anions together with that of urea and guanidine hydrochloride on the fluorescence emissions of spinach chloroplasts at 77 °K are presented in Table I. In the table, *f*-685 means the relative intensity of the fluorescence emission measured at 685 nm (read from an uncorrected fluorescence emission spectrum). Where the F-695 emission has no distinct maximum in the spectrum, *f*-695 likewise represents the relative intensity of the fluorescence emission at 695 nm. For the sake of convenience, however, where the second band (F-695 or F-700) has a distinct emission maximum in the uncorrected emission spectrum, *f*-695 means

TABLE I

EFFECTS OF INORGANIC ANIONS ON THE FLUORESCENCE EMISSION OF SPINACH CHLOROPLASTS AT 77 °K

Chloroplast preparations were suspended in 50 mM Tris–HCl (pH 7.2) in water. Fluorescence intensities were measured in the absence (— phen) and in the presence (+ phen) of 1 mM 1,10-phenanthroline. See text for an explanation of the *f*-695/*f*-685 ratio. +phen/—phen represents the ratio of *f*-695/*f*-685 in the presence of 1,10-phenanthroline to that in the absence of 1,10-phenanthroline.

Substance (100 mM)	*f*-695/*f*-685		
	—phen	+phen	+phen/—phen
	1.16	1.20	1.03
Guanidine hydrochloride	0.93	2.33	2.51
Urea	0.92	1.58	1.72
KSCN	0.77	1.37	1.78
KI	0.89	1.26	1.42
$NaClO_4$	0.68	0.97	1.43
KNO_3	1.04	1.08	1.04
KBr	0.98	1.02	1.04
KCl	1.06	1.05	0.99
$KClO_3$	1.10	1.14	1.04
K_2SO_4	0.98	1.00	1.02
Potassium acetate	0.91	0.92	1.01

the fluorescence intensity at the peak wavelength. The ratio of *f*-695 to *f*-685 was decreased by many compounds such as urea, guanidine hydrochloride, and KSCN principally because of the partial quenching of the F-695 emission (this becomes obvious by fourth-derivative analysis of the emission spectrum). On the other hand, the activation of the F-695 emission accompanying the red-shift of the emission maximum in the presence of 1,10-phenanthroline (transformation of F-695 into F-700), is caused only by KSCN, KI, $NaClO_4$ and by the compounds of the urea–guanidine class. The degree of activation produced by these inorganic anions was in general smaller than that caused by urea or guanidine hydrochloride. Inorganic anions such as NO_3^-, Br^-, Cl^-, ClO_3^- and SO_4^{2-} were inactive in the activation. In general, the order of effectiveness in the activation in the presence of 1,10-phenanthroline on the part of these inorganic anions and of compounds of the urea–guanidine class was as follows;

Guanidine hydrochloride > methylguanidine hydrochloride > thiourea > urea > *N*-methylurea > dimethylurea, urea > SCN^- > I^- > ClO_4^-

As mentioned above, the inorganic anions and the compounds of the urea–guanidine class cause a partial quenching of the F-695 emission in the absence of 1,10-phenanthroline (Fig. 3 and Table I), and cause a transformation of F-695 into F-700 in the presence of 1,10-phenanthroline. The quenching, however, seems not to be a prerequisite for the transformation, for two reasons. First, there is some discrepancy in the concentration dependence between the quenching and the transformation, as shown in Fig. 4. The quenching of the F-695 emission and its transformation are saturated in respect to the concentration of urea at about 100 mM and 1 M, respectively. The effective concentrations in both quenching and transformation were

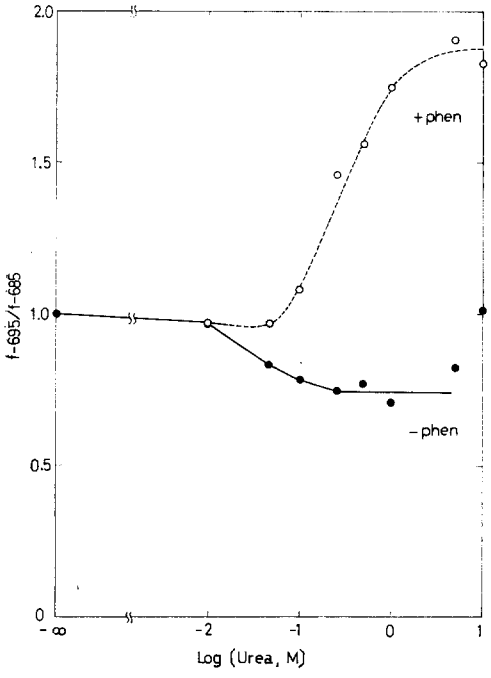

Fig. 4. Effect of 1,10-phenanthroline on the *f*-695/*f*-685 ratio of spinach chloroplasts, at 77 °K, in the presence of varying concentrations of urea. See text for an explanation of the *f*-695/*f*-685 ratio. Fluorescence intensities were measured in the presence (+phen) and in the absence (−phen) of 1 mM 1,10-phenanthroline. Chloroplast preparations were suspended in 50 mM Tris–HCl (pH 7.2) in water.

somewhat variable from reagent to reagent, shifting to lower concentrations in the case of guanidine hydrochloride. Secondly, the magnitude of the quenching has no relationship to the transformation. For example, inorganic anions such as KSCN and $NaClO_4$ have a larger activity in the quenching, but have a lower activity in the transformation, as compared with guanidine hydrochloride (Table II). In addition, methyl-substituted ureas such as *N*-methylurea and dimethylurea, or methylguanidine hydrochloride had an activity nearly equal to that of urea or guanidine hydrochloride, respectively, in the case of quenching, but had a much reduced activity in the transformation. Thus, though the quenching and the transformation are probably intimately related, they represent a separate action of those reagents.

The effective concentrations of the compounds of the urea–guanidine class and of the inorganic anions such as ClO_4^-, I^- and SCN^- are intermediate between those which cause quenching of the F-695 emission and those which cause unfolding or denaturation of proteins; the latter usually seems to be a cooperative reaction occurring at high concentrations (several molar) [39].

Derivatives of 1,10-phenanthroline

Derivatives of 1,10-phenanthroline and other monofunctional or bifunctional ligands were examined for efficiency in activity of the transformation of F-695 into

TABLE II

ACTIVATION OF F-695 EMISSION BY SOME DERIVATIVES OF 1,10-PHENANTHROLINE AND A FEW OTHER SUBSTANCES

Chloroplast preparations from spinach were suspended in a medium containing 50 mM Tris–HCl (pH 7.2), 100 mM guanidine hydrochloride, ethanol (final concn 1 %) and water. The degree of activation (and spectral shift) of the F-695 emission was expressed by ++, +, ±, and − (no activation). pK_a, acid dissociation constant at 25 °C are cited from the refs; pI_{50}, $-\log_{10}$ of molar concentration of substances giving 50 % inhibition of DCIP photoreduction.

Substance	Activation	pK_a	pI_{50}	ref.
Phenanthroline derivatives				
2,9-Dimethyl-1,10-phenanthroline	−	6.15	< 2	40
4,7-Dimethyl-1,10-phenanthroline	±	5.94	5.9	40
2,9-Dimethyl-4,7-diphenyl-1,10-phenanthroline	−	5.80*	< 2	41
5-Methyl-1,10-phenanthroline	+	5.26	4.2	40
4,7-Diphenyl-1,10-phenanthroline sulfonate	−	5.20*	< 2.5	41
1,10-Phenanthroline	++	4.92	4.4	42
4,7-Diphenyl-1,10-phenanthroline	−	4.84	< 3.5	43
5-Nitro-1,10-phenanthroline	−	3.33	< 3	44
Miscellaneous				
Pyridine	−	5.21		
2,2′-Dipyridine	−	4.44		
4,4′-Dipyridine	−			
1,3-Diazine	−			
1,4-Diazine	−			
2,4,6-Tris-(2-pyridyl)-s-triazine	−			
o-Phenylenediamine	−			
Diamino benzidine-HCl	−			

* Temperature of the measurements is not specified.

F-700, in the presence of 100 mM guanidine hydrochloride. The names of the compounds tested are listed in Table II; the pK_a values [40–44] at 25 °C and pI_{50} values [Satoh, K., unpublished] for the inhibition of 2,6-dichlorophenolindophenol (DCIP) photoreduction at room temperature of these reagents are supplied on the right columns of the table. Among these compounds the only active ones are 1,10-phenanthroline, 5-methyl-1,10-phenanthroline, and 4,7-dimethyl-1,10-phenanthroline (Fig. 5). 5-Methyl-1,10-phenanthroline or 4,7-dimethyl-1,10-phenanthroline in the absence of guanidine hydrochloride had no effect on the fluorescence emission spectrum of chloroplasts at 77 °K (data not shown), as 1,10-phenanthroline (for example, see Fig. 3 or Table I). Methyl substitution, which in general decreases water solubility and increases lipid solubility, greatly reduced the transforming activity; 4,7-dimethyl-1,10-phenanthroline activated the F-695 emission only slightly (Fig. 5). This state of affairs is quite different from that of the inhibition of the photosynthetic electron transport chain by these reagents, where 4,7-dimethyl-1,10-phenanthroline, the least active compound in the case of transformation shown in Fig. 5, is the most potent inhibitor, suggesting a different kind of action of 1,10-phenanthroline on Photosystem II. Other bifunctional ligands such as 2,2′-dipyridine, 4,4′-dipyridine, 1,3-diazine, and 1,4-diazine as well as pyridine were all inactive.

Metal ions which form a phenanthroline complex with a high stability constant

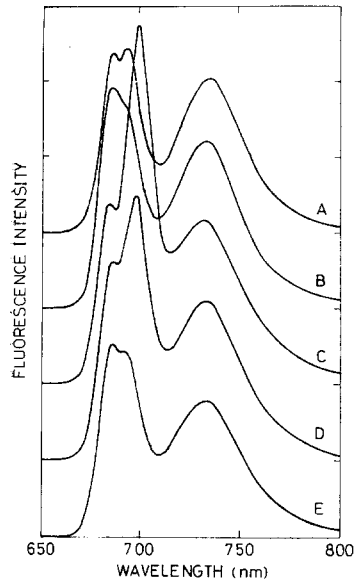

Fig. 5. Fluorescence emission spectra of spinach chloroplasts, at 77 °K, in the presence of guanidine hydrochloride and some derivatives of 1,10-phenanthroline: A, no additions; B, 100 mM guanidine hydrochloride; C, 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline; D, 100 mM guanidine hydrochloride and 1 mM 5-methyl-1,10-phenanthroline; E, 100 mM guanidine hydrochloride and 1 mM 4,7-dimethyl-1,10-phenanthroline. Chloroplast preparations were suspended in a medium containing 50 mM Tris–HCl (pH 7.2) and 1 % ethanol. See legend to Fig. 3 for further explanation. Band width, 5.0 nm.

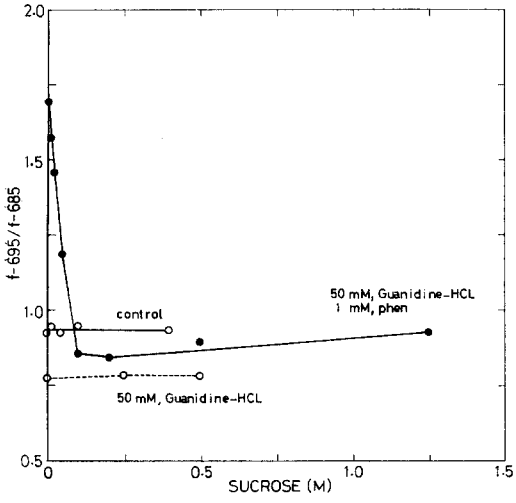

Fig. 6. Effect of varying concentrations of sucrose on the *f*-695/*f*-685 ratio of spinach chloroplasts at 77 °K: ○–○, and ——, no additions; ○–○, and ---, 50 mM guanidine hydrochloride; ●–●, 50 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. See legend to Fig. 4 for further explanation.

such as Ni^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}, Hg^{2+} and Mn^{2+} reversed the transformation (cf. Kautsky et al. [45]), while those with a low stability constant such as Mg^{2+}, Ca^{2+}, Al^{3+}, and B^{2+} had no effect. Thus, a metal complex of 1,10-phenanthroline such as ferroin did not act as a transforming agent.

Inhibition of transformation

Three classes of compounds prevented the transformation of F-695 into F-700. Fig. 6 shows the *f*-695/*f*-685 ratio, represented in the manner described for Table I, for fluorescence emissions in the presence of sucrose. The *f*-695/*f*-685 ratio is not affected by sucrose in the case of the control or in the presence of 50 mM guanidine hydrochloride, but is markedly changed with sucrose concentrations in the presence of 50 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. The *f*-695/*f*-685 ratio in the absence of sucrose in Fig. 6 is about 1.7. The addition of sucrose markedly reduced the ratio and finally eliminates the activation and the spectral shift at about 100 mM (i.e. much lower than that used as an isotonic solution for the extraction of chloroplasts, typically 400 mM), but the quenching caused by guanidine hydrochloride remains (this was clearly seen in the fluorescence emission spectrum). Higher concentrations of sucrose, e.g. 1.25 M, also eliminated this quenching, but the affairs are somewhat complex. Glucose also caused an identical effect as sucrose on the fluorescence emission spectrum of spinach chloroplasts at 77 °K.

Fig. 7 shows the effect of varying concentrations of NaCl on uncorrected fluorescence emission spectra of spinach chloroplasts at 77 °K, in the presence of 50 mM guanidine hydrochloride (Fig. 7-A) and in the presence of 50 mM guanidine

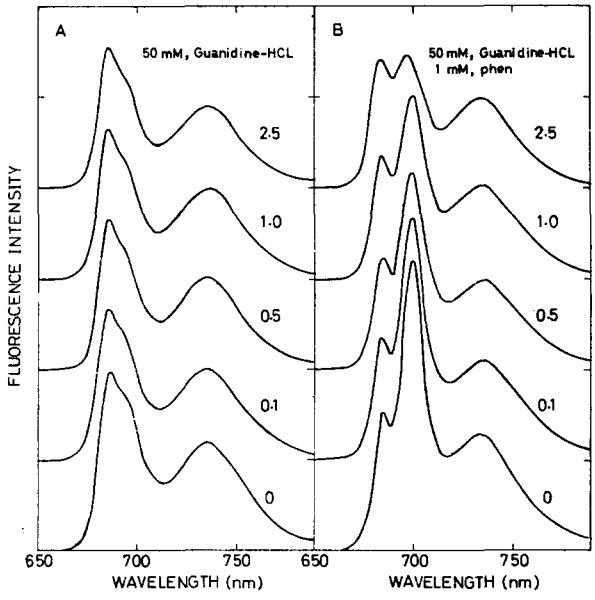

Fig. 7. Fluorescence emission spectra of spinach chloroplasts, at 77 °K, in the presence of varying concentrations of NaCl: A, 50 mM guanidine hydrochloride; B, 50 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. The number beside each solid line indicates the molar concentration of NaCl in the sample. See legend to Fig. 3 for further explanation.

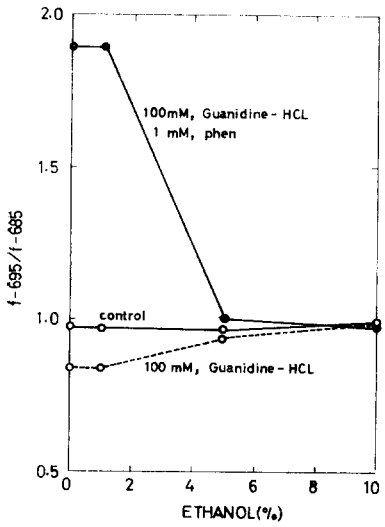

Fig. 8. Effect of varying concentrations of ethanol on the *f*-695/*f*-685 ratio of spinach chloroplasts at 77 °K: ○–○, no additions; ○- - -○, 100 mM guanidine hydrochloride; ●–● 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. See legend to Fig. 4 for further explanation.

hydrochloride and 1 mM 1,10-phenanthroline (Fig. 7-B). The fluorescence emission spectra for the control sample (data not shown) and those in the presence of 50 mM guanidine hydrochloride were only slightly affected by NaCl. However, in the presence of 50 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline, the transformation of F-695 into F-700 was markedly reduced with increasing concentrations of NaCl in the concentration range examined; 2.5 M of NaCl seems to be insufficient for complete inhibition, as the spectrum can be expected to coincide with that in the presence of guanidine hydrochloride plus NaCl (Fig. 7-A). This salt effect is not ion specific; both KCl and Na_2SO_4 can substitute for NaCl.

In contrast to the effect of sugars and salts (where the inhibition is specific for transformation, but not for quenching), ethanol [32], glycerine, ethyleneglycol, and dimethylsulfoxide (the compounds which are often used as a solvent for spectroscopic observation at liquid nitrogen temperature or used as a protective agent for storage of chloroplasts at liquid nitrogen temperature) simultaneously diminished both the transformation and the quenching of F-695. The data for the inhibitions in the case of ethanol are shown in Fig. 8. The effective concentrations for the inhibitions both of the transformation and of the quenching are identical in the case of ethanol and also in the cases of other solvents: 1–5 %.

The inhibitions caused by sugars, salts, and by organic solvents were reversible. The actual causes of the inhibitions by these reagents are difficult to understand at present, but a few things can be ruled out: the osmotic effect and the effect of ionic strength is not primarily concerned (see Discussion).

The nature of F-700

F-695 fluorescence is photoinitiated as in the case of the F-685 emission at room temperature and at liquid nitrogen temperature. This phenomenon has been

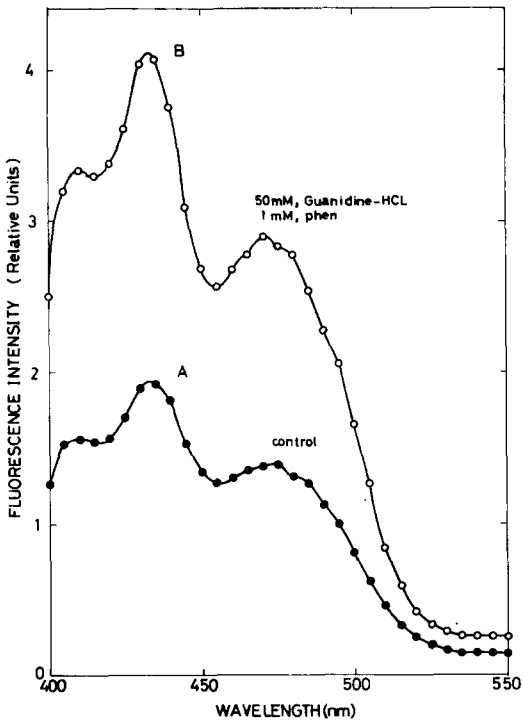

Fig. 9. Fluorescence excitation spectra of spinach chloroplasts, at 77 °K, in the absence (A) and in the presence (B) of 50 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. Fluorescence was measured at 702 nm (half-band width, 9.0 nm). See legend to Fig. 1 for further explanation.

taken as direct evidence supporting the interpretation that this pigment participates in the primary photochemical act in photosynthesis [2, 10, 11]. F-700 fluorescence also involves an induction phenomenon at the onset of illumination [32], indicating that the pigment is still associated with the photosynthetic electron transport chain.

Fig. 9 shows fluorescence excitation spectra, from 400–550 nm, measured at 702 nm (half-band width, 9.0 nm) at 77 °K of spinach choroplasts in the presence or in the absence of 50 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. The spectrum in the absence of guanidine and phenanthroline corresponds with the excitation spectrum for the F-695 emission and that in the presence of those reagents for the F-700 emission. The shape of the excitation spectrum for the F-700 emission in the figure is quite similar to that for the F-695 emission, and they resemble the excitation spectrum for the F-685 emission (data not shown); i.e. a relatively higher efficiency of energy transfer from chlorophyll *b* and carotenoids than those in the F-735 emission. This fact indicates that the pigment system which gathers excitation energy (bulk pigments) is identical for F-685, F-695 and F-700; suggesting that F-700 is still to be associated with Pigment-system II of photosynthesis.

F-695 emission is not visible at temperatures above 150 °K but markedly increases below this temperature [13, 28–31]. The steep temperature dependence of the fluorescence emission over a fairly short temperature range at low temperatures has been one of the unique properties of the F-695 emission [30]. This unique pro-

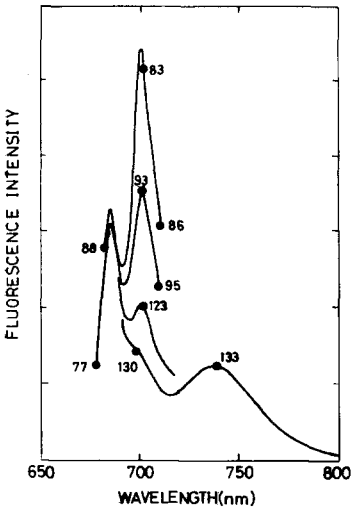

Fig. 10. Fluorescence emission spectra of spinach chloroplasts in the presence of 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline at varying temperatures. The temperatures of sample were gradually changed during the spectral scanning. The number beside each point indicates the temperature (°K) of the sample at the measurement. See legend to Fig. 3 for further explanation.

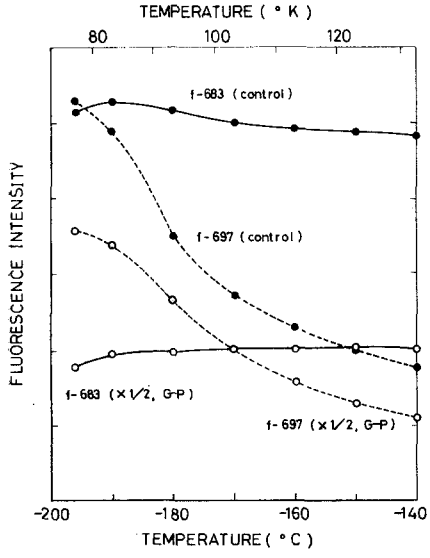

Fig. 11. Fluorescence intensities of spinach chloroplasts at 683 nm (——) and at 697 nm (---) plotted against temperatures of the measurement: ●, no additions; ○, 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. The intensities of fluorescence in the presence of guanidine hydrochloride and 1,10-phenanthroline are plotted with a reduced scale (× 0.5). See legend to Fig. 3 for further explanation.

perty is probably closely related to the molecular environments of the fluorophores of the emission component in the chloroplast membranes, and the increase in low temperatures seems to be due to an increase in the fluorescence yield rather than in energy transfer. Fig. 10 shows the fluorescence emission spectra of spinach chloroplasts in the presence of 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline measured at different temperatures (the temperatures of the sample were measured with a calibrated thermocouple and the temperatures of the sample were changed by introducing liquid nitrogen-cooled air). The temperature dependence of fluorescence intensities measured at 683 nm (F-685) and at 697 nm (F-695 or F-700) are shown in Fig. 11. From Figs 10 and 11, it is evident that the steep temperature dependence of the F-695 emission is evidently preserved in F-700. This also supports the idea that F-700, although it has a different emission-maximum wavelength and a different emission intensity, is a variant of F-695 since basic properties other than spectroscopic properties are identical with F-695.

Generality of the phenomena

The presence of the F-695 band in the fluorescence emission spectra at 77 °K and the transformation of F-695 into F-700 as induced by guanidine hydrochloride and 1,10-phenanthroline were examined in chloroplast preparations from 11 higher plants and two species of green algae. These include *Spinacia oleracea*, *Phytolacca americana*, *Rumex japonicus*, *Beta vulgaris* var. *Cicla*, *Lolium multiforum*, *Trifolium repens*, *Miscanthus sinensis*, *Vicia sativa*, *Erigeron linifolius*, *Petroselinum sativum*, *Lactuca sativa*, *Chlamydomonas reinhardi* and *Ulva pertusa*. A distinct emission peak of F-695 was observed in *Spinacia*, *Phytolacca*, *Rumex*, *Beta*, and two species of algae in the uncorrected spectra in these experiments. The presence of an F-695 emission band in the seven other higher plants was noticed as a shoulder or as an asymmetry in the F-685 band. On addition of 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline, a distinct emission maximum at a point between 695 and 700 nm becomes manifest in the latter cases. Thus, the transformation of F-695 into F-700 as well as the presence of F-695 seems to be a general phenomenon in green plants, indicating that F-695 exists in a similar molecular environment in all green plants.

An interesting fact is that F-695 is partly activated by 1,10-phenanthroline

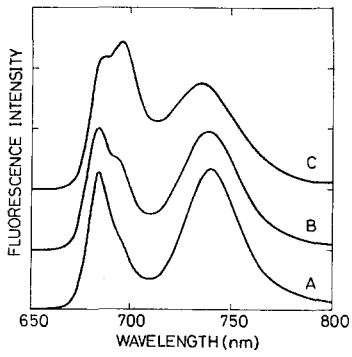

Fig. 12. Fluorescence emission spectra of *Lolium* chloroplasts, at 77 °K, in the presence of 1,10-phenanthroline: A, no additions; B, 1 mM 1,10-phenanthroline; C, 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. See legend to Fig. 3 for further explanation. Band width, 5.0 nm.

alone (without guanidine hydrochloride) in the case of *Lolium* (Fig. 12). This may indicate a difference in the molecular environments of F-695 in this plant from those of the other materials. This fact also supports the idea 1,10-phenanthroline is the material which causes the transformation and that the compounds of the urea–guanidine class or inorganic anions such as SCN^-, I^- and ClO_4^- assist the reaction of 1,10-phenanthroline on F-695 by changing the molecular environments of the pigment in chloroplast membranes.

DISCUSSION

At the expense of an intensity decrease in the F-695 emission, a new fluorescence band with an emission maximum at 700 nm (F-700) arises in chloroplasts by the concomitant addition of chaotropic reagents (see latter part of this Discussion) and 1,10-phenanthroline. The new emission band has two distinct fluorescence characteristics, that is, the wavelength of the emission maximum and the fluorescence intensity, but still retains the principal characteristics of F-695; i.e. a steep temperature dependence at low temperatures, transient phenomena at the onset of illumination [32], and an excitation spectrum of the Photosystem II type. Thus, F-700 can be regarded as a transformed species of F-695. It can be expected that changes in the fluorescent energy level in the fluorophores may bring about corresponding changes in the absorption of the components. Detailed analysis of changes in low-temperature absorption and fluorescence excitation spectra, as induced by 1,10-phenanthroline in the presence of chaotropic reagents, provided no informations on the problem (data not shown). This probably supports the assumption [2, 3, 5, 10, 11] that the pigment F-695 is only a minor component which accepts excitation energy from bulk pigments.

The fluorescence intensity of F-700 at a saturating concentration of chaotropic reagents and 10 mM, 1,10-phenanthroline (nearly its maximum solubility in water) was about twice that of F-695 in the control sample. The increase in fluorescence intensity as a result of additions may be an increase in: (a) absorption, (b) fluorescence yield, or (c) energy transfer to the emitting species. The changes in the absorption spectrum by the addition of 1,10-phenanthroline (in the presence of chaotropic reagents) are too small to account for the marked increase in F-695 (F-700) and are inconsistent with a decrease in the F-685 emission. The increase in the fluorescence intensity may be partly due to the enhanced excitation transfer from the bulk chlorophylls (F-685) to the pigments (F-700), because the F-685 emission is partly reduced along with the activation of the F-695 (F-700) emission. The mechanism of enhancement of the excitation transfer can be explained by changes in the trap depth in a photosystem (displacement of absorption bands) as proposed for the explanation of the temperature dependence of the F-695 emission by Govindjee et al. [46]. The main part of the fluorescence increase is probably due to changes in fluorescence efficiency (relative decrease in the probability of internal conversion, intersystem crossing, or photochemistry). The presence of similar transient phenomena [32] at the onset of illumination for the F-700 band as for the F-695 band, however, seems to reduce the possibility that the suppression of photochemistry is the main cause of the increase in fluorescence efficiency.

The term "chaotropic agents" was used by Hatefi and Hanstein [47] to de-

scribe a group of compounds which, by altering the bulk properties of water, can cause membrane-bound proteins to become soluble. They include ClO_4^-, SCN^-, I^-, urea and guanidine hydrochloride [47]. The specificity and the order of effectiveness of the compounds in transforming the F-695 into F-700 reported in this paper seem largely to coincide with those of the chaotropic reagents in effecting the salting-in of organic molecules [47], the resolution of NADH–CoQ reductase from mitochondrial membranes [48, 49], and the dissociation of the antigen–antibody complex [50]. Thus, the role of the compounds of the urea-guanidine class and of inorganic anions such as SCN^-, I^- and ClO_4^- in effecting the process of transformation of F-695 into F-700 can be expected to be similar in nature to those. The concentration of chaotropic reagents in effecting the transformation is lower than that which causes denaturation of proteins, suggesting that the transformation does not involve gross changes in the backbone structure of the membrane proteins. In chloroplast membranes, F-695 fluorophores or some compounds intimately associated with fluorophores appear to be protected by a hydrophobic sheath of lipids and structural proteins with entirely different characteristics from F-685 and F-735. Such a structure is consistent with the fact that 1,10-phenanthroline does not readily react, for example in spinach chloroplasts, with the reactive moiety of the system, but does do so in the presence of chaotropic reagents. The inhibition of electron transport in chloroplasts by chaotropic reagents was reported by Lozier et al. [51].

It is of interest to compare the effect of salts on the transformation of F-695 into F-700 with that of NaCl on the EPR spectrum of spinach ferredoxin [52, 53]. In both cases the changes were observed at liquid nitrogen temperature. In the case of ferredoxin, urea-induced changes in the EPR spectrum were inhibited by the presence of relatively higher concentrations of salt in a parallel fashion with the changes in the absorption spectrum and the CD spectrum measured at room temperature. This indicates that, at least in spinach ferredoxin in high concentrations of salt, the chromophore and EPR center are only marginally perturbed by the presence of a potential denaturant. Probably a similar mechanism of salt action as in the case of spinach ferredoxin can be expected in the case of the transformation of F-695 into F-700. In the presence of a low concentration of sucrose (approx. 100 mM), F-695 is stable and retains its ability to fluoresce at about 695 nm in the presence of 100 mM guanidine hydrochloride and 1 mM 1,10-phenanthroline. A significant effect of sugars on proteins or membrane conformation has been reported in the cases of chlorophyllide-holochrome [54], β-lactoglobulin A [55], and mitochondrial membranes [56]. The most plausible explanation of this sugar effect and probably salt effect, is that sugar, and probably salt, by reducing the activity of water (dehydration effect), leads to a change in the structure of chloroplast membranes, which in turn prevents the reaction of 1,10-phenanthroline on F-695. A similar but somewhat different mechanism may exists in the cases of alcohols and dimethylsulfoxide.

As shown in Fig. 5 and Table II, there is no paralellism between the activity of transformation and the pKa values at 25 °C of phenanthroline derivatives; most 1,10-phenanthroline derivates except for 4,7-diphenyl-1,10-phenanthroline and 5-nitro-1,10-phenanthroline, have larger pK_a values than that of 1,10-phenanthroline, but have lower or no activity in the transformation. Solubility in water or in lipids does not seem to be a primary factor for the activity; alkyl and aryl substituents (2,9-dimethyl-, 4,7-dimethyl-, 4,7-diphenyl-, etc.) tend to increase the lipid solubility

and the introduction of a readily ionizable substituent group such as sulfonic acid increase the water solubility, but both of these have a lower activity than 1,10-phenanthroline or sometimes none at all, in the transformation of F-695 into F-700. The remaining possibility is a complex steric effect (relating to 2,9-positions and/or 4,7-positions) or a combination of various factors. The specificity of 1,10-phenanthroline in the interaction is a matter for further investigation.

1,10-Phenanthroline has been shown to be a potent inhibitor of photosynthetic reactions both in plants [45] and in bacterial systems [57–59]. 1,10-Phenanthroline shifts the midpoint potentials of the primary electron acceptor in bacterial systems [59]. The action of 1,10-phenantrholine in the transformation of F-695 into F-700 however, is different from the inhibition of photosynthetic electron transport in three respects: (a) the potency of phenanthroline-derivatives in effecting the inhibition of photosynthetic electron transport (Satoh, K., unpublished) and in the transformation differs greatly; in the case of the inhibition of electron transport, 4,7-dimethyl-1,10-phenanthroline (pI_{50}, 5.9) is the most potent inhibitor, and 1,10-phenanthrline (pI_{50}, 4.4) and 5-methyl-1,10-phenanthroline (pI_{50}, 4.2) follow this, while in the case of the transformation of F-695 into F-700, the order of effectiveness is as follows, 1,10-phenanthroline $>$ 5-methyl-1,10-phenanthroline $\geq$ 4,7-dimethyl-1,10-phenanthroline; (b) the concentrations of reagents in effecting the inhibition of electron transport ($10^{-4.4}$ M, the pI_{50} value for the inhibition of DCIP photoreduction [Satoh, K., unpublished] and in the transformation (10^{-4}–10^{-2} M) are very different; and (c) the presence of chaotropic reagents is necessary in the case of the transformation in spinach chloroplasts, while in the case of inhibition of the electron transport, the presence of these reagents had no effect on the percent inhibition. Thus, the action of 1,10-phenanthroline in the transformation of F-695 into F-700 is an as yet unrecognized reaction of the reagent on the photosynthetic system of chloroplasts. It is of interest to describe the results of Clayton et al. [58] on the bacterial systems, where, besides the inhibition of electron transport from primary to secondary acceptors, 1,10-phenanthroline inhibited the quantum efficiency of the photochemical oxidation of P890. The inhibition was more severe at higher concentrations of a detergent, lauryldimethylamine oxide [58].

The transformation of F-695 into F-700 can be explained by two different models: (a) 1,10-phenanthroline interacts directly with fluorophores of F-695 to convert the fluorescent energy levels and the fluorescence efficiency; (b) F-695 is closely associated with protein in chloroplast membranes and the reaction of 1,10-phenanthroline, directly or via metal ion, on the protein dramatically changes the interaction of protein–fluorophore or fluorophore– fluorophore; this produces some changes in the fluorescent energy levels and the fluorescence efficiency of F-695. The available data about the action of 1,10-phenanthroline in the transformation are insufficient to prove a definite model at present, but the interaction of the fluorophore of F-695 with some specific protein in most likely.

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REFERENCES

1 Litvin, F. F., Krasnovsky, A. A. and Rikhireva, C. T. (1960) Dokl. Acad. Nauk. SSSR 135, 1528–1531
2 Kok, B. (1963) Photosynthetic Mechanism of Green Plants (Kok, B. and Jagendorf, A. T., eds), p. 45, Natl. Acad. Sci.–Natl. Res. Council, Washington, D. C.
3 Govindjee (1963) Photosynthetic Mechanism of Green Plants (Kok, B. and Jagendorf, A. T., eds), p. 318, Natl. Acad. Sci.–Natl. Res. Council. Washington, D. C.
4 Brody, S. S. and Brody, M. (1963) Photosynthetic Mechanism of Green Plants (Kok, B. and Jagendorf, A. T., eds), p. 455, Natl. Acad. Sci.–Natl. Res. Council. Washington, D. C.
5 Bergeron, J. A. (1963) Photosynthetic Mechanism of Green Plants (Kok, B. and Jagendorf, A. T., eds), p. 527, Natl. Acad. Sci.–Natl. Res. Council, Washington, D. C.
6 Goedheer, J. C. (1964) Biochim. Biophys. Acta 88, 304–317
7 Murata, N., Nishimura, M. and Takamiya, A. (1966) Biochim. Biophys. Acta 126, 234–243
8 Goedheer, J. C. (1968) Biochim. Biophys. Acta 153, 903–906
9 Goedheer, J. C. (1970) Photosynthetica 4, 97–106
10 Murata, N. (1968) Biochim. Biophys. Acta 162, 106–121
11 Donze, M. and Duysens, L. N. M. (1969) Progress in Photosynthesis Research (Metzner, H., ed.), Vol. 2, p. 991, I.U.B. Sci., Tübingen
12 Krey, A. and Govindjee (1964) Proc. Natl. Acad. Sci. U.S. 52, 1568–1572
13 Krey, A. and Govindjee (1966) Biochim. Biophys. Acta 120, 1–18
14 Boardman, N. K., Thorne, S. W. and Anderson, J. M. (1966) Proc. Natl. Acad. Sci. U.S. 56, 586–593
15 Kok, B. and Rurainski, H. J. (1966) Biochim. Biophys. Acta 126, 584–587
16 Cederstrand, C. N. and Govindjee (1966) Biochim. Biophys. Acta 120, 177–180
17 Ke, B. and Vernon, L. P. (1967) Biochemistry 6, 2221–2226
18 Boardman, N. K. and Thorne, S. W. (1969) Biochim. Biophys. Acta 189, 294–297
19 Bril, C., van der Horst, D. J., Poort, S. R. and Thomas, J. B. (1969) Biochim. Biophys. Acta 172, 345–348
20 Boardman, N. K. (1972) Biochim. Biophys. Acta 283, 469–482
21 Mohanty, P., Braun, B. Z., Govindjee and Thornber, J. P. (1972) Plant and Cell Physiol. 13, 81–91
22 Okayama, S. and Butler, W. L. (1972) Plant Physiol. 49, 769–774
23 Mohanty, P., Mar, T. and Govindjee (1971) Biochim. Biophys. Acta 253, 213–221
24 Jacobs, R. and Brody, S. S. (1972) Biochim. Biophys. Acta 267, 341–347
25 Okayama, S. and Butler, W. L. (1972) Biochim. Biophys. Acta 267, 523–529
26 Butler, W. L. (1972) Proc. Natl. Acad. Sci. U.S. 69, 3420–3422
27 Butler, W. L., Visser, J. W. M. and Simons, H. L. (1973) Biochim. Biophys. Acta 292, 140–151
28 Govindjee and Yang, L. (1966) J. Gen. Physiol. 49, 763–780
29 Broyde, S. B. and Brody, S. S. (1966) Biophys. J. 6, 353–366
30 Cho, F. and Govindjee (1970) Biochim. Biophys. Acta 205, 371–378
31 Goedheer, J. C. and Verhüllsdonk, C. A. H. (1970) Biochim. Biophys. Res. Commun. 39, 260–266
32 Satoh, K. (1972) Plant Cell Physiol. 13, 23–34
33 Brody, M., Nathanson, B. and Cohen, W. S. (1969) Biochim. Biophys. Acta 172, 340–342
34 Nathanson, B. and Brody, M. (1970) Photochem. Photobiol. 12, 469–479
35 Satoh, K. (1970) Plant and Cell Physiol. 11, 15–27
36 Morrey, J. R. (1968) Anal. Chem. 40, 905–914
37 Butler, W. L. and Hopkins, D. W. (1970) Photochem. Photobiol. 12, 439–450
38 Butler, W. L. and Hopkins, D. W. (1970) Photochem. Photobiol. 12, 451–456
39 Tanford, C. (1968) Adv. Protein Chem. 23, 121–282
40 Yasuda, M., Sone, K. and Yamasaki, K. (1956) J. Phys. Chem. 60, 1667–1668
41 Blair, D. and Diehl, H. (1961) Talanta 7, 163–174

42 Yamasaki, K. and Yasuda, M. (1956) J. Am. Chem. Soc. 78, 1324
43 Schilt, A. A. and Smith, G. F. (1956) J. Phys. Chem. 60, 1546–1548
44 Banks, C. V. and Bystroff, R. C. (1959) J. Am. Chem. Soc. 81, 6153–6158
45 Kautsky, H., Appel, W. and Amann, H. (1960) Biochem. Z. 332, 277–292
46 Govindjee, Papageorgiou, G. and Rabinowitch, E. (1967) Fluorescence, Theory, Instrumentation and Practice (Guilbault, G. G. ed.) p. 511, Marcel Deckker, Inc., New York
47 Hatefi, Y. and Hanstein, W. G. (1969) Proc. Natl. Acad. Sci. U.S. 62, 1129–1136
48 Hatefi, Y. and Stempel, K. E. (1969) J. Biol. Chem. 244, 2350–2357
49 Hatefi, Y., Davis, K. A. and Hanstein, W. G. (1970) Arch. Biochem. Biophys. 137, 286–287
50 Dandliker, W. B., Alonso, R., de Saussure, V. A. and Kierszenbaum, H. C. (1967) Biochemistry 6, 1460–1467
51 Lozier, R., Baginsky, M. and Butler, W. L. (1971) Photochem. Photobiol. 14, 323–328
52 Peterring, D. H. and Palmer, G. (1970) Arch. Biochem. Biophys. 141, 456–464
53 Cammack, R., Rao, K. K. and Hall, D. O. (1971) Biochem. Biophys. Res. Commun. 44, 8–14
54 Mathis, P. and Sauer, K. (1972) Biochim. Biophys. Acta 267, 498–511
55 Clement-Metral, J. and Yon, J. (1968) Biochim. Biophys. Acta 160, 340–359
56 Zimmer, G., Keith, A. D. and Packer, L. (1972) Arch. Biochem. Biophys. 152, 105–113
57 Parson, W. W. and Case, G. O. (1970) Biochim. Biophys. Acta 205, 232–245
58 Clayton, R. K., Szutz, E. Z. and Fleming, H. (1972) Biophys. J. 12, 64–79
59 Jackson, J. B., Cogdell, R. J. and Crofts, A. R. (1973) Biochim. Biophys. Acta 292, 218–225